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Melissa DeYoung
Portland State University

Michelle Thayer
Portland State University

John Van der Oost
Wageningen University

Kenneth M. Stedman
Portland State University, kstedman@pdx.edu

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RESEARCH LETTER

Growth phase-dependent gene regulation *in vivo* in *Sulfolobus solfataricus*

Melissa DeYoung[1], Michelle Thayer[1], John van der Oost[2] & Kenneth M. Stedman[1]

[1]Biology Department and Center for Life in Extreme Environments, Portland State University, Portland, OR, USA; and [2]Laboratory of Microbiology, Wageningen University and Research Center, Wageningen, The Netherlands

Correspondence: Kenneth Stedman, Biology Department and Center for Life in Extreme Environments, Portland State University, PO Box 751, Portland, OR 97207, USA. Tel.: +1 503 725 3253; fax: +1 503 725 3888; e-mail: kstedman@pdx.edu

Present address: Michelle Thayer, Department of Microbiology, Immunology and Molecular Genetics, UCLA, Los Angeles, CA 90095, USA.

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Abstract

Ribosomal genes are strongly regulated dependent on growth phase in all organisms, but this regulation is poorly understood in Archaea. Moreover, very little is known about growth phase-dependent gene regulation in Archaea. SSV1-based *lacS* reporter gene constructs containing the *Sulfolobus* 16S/23S rRNA gene core promoter, the *TF55*α core promoter, or the native *lacS* promoter were tested in *Sulfolobus solfataricus* cells lacking the *lacS* gene. The 42-bp 16S/23S rRNA gene and 39-bp *TF55*α core promoters are sufficient for gene expression in *S. solfataricus*. However, only gene expression driven by the 16S/23S rRNA gene core promoter is dependent on the culture growth phase. This is the smallest known regulated promoter in *Sulfolobus*. To our knowledge, this is the first study to show growth phase-dependent rRNA gene regulation in Archaea.

Introduction

Regulation of rRNA transcription is critical for cellular life and has been investigated extensively in Bacteria and Eukarya, where it is tightly regulated by multiple and overlapping mechanisms including growth phase-dependent regulation (Nomura, 1999; Schneider *et al*., 2003). However, little is known about rRNA transcriptional regulation in Archaea. rRNA genes in Archaea are frequently linked, containing the 23S rRNA gene downstream of the 16S rRNA gene (http://archaea.ucsc.edu). *Sulfolobus solfataricus* and *Sulfolobus shibatae* contain single 16S/23S rRNA gene operons that have been previously studied *in vivo* and *in vitro* (Reiter *et al*., 1990; Qureshi *et al*., 1997).

The basal transcriptional apparatus of Archaea is similar to that of Eukaryotes (reviewed in Bartlett, 2005). However, most putative transcriptional regulators are homologues of bacterial transcription factors and appear to act similarly, by either preventing or facilitating the assembly of the transcriptional preinitiation complex (Bell, 2005; Peng *et al*., 2011). How the regulators function *in vivo* is unclear partly due to the lack of efficient genetic systems for many Archaea. The majority of transcriptional regulation analyses in Archaea, particularly thermoacidophilic Archaea, have been performed *in vitro*. This is changing with the development of genetic tools for *S. solfataricus* (Wagner *et al*., 2009), *Sulfolobus islandicus* (Peng *et al*., 2011), and *Sulfolobus acidocaldarius* (Berkner *et al*., 2010). Regulation of rRNA transcription remains particularly cryptic, as most current approaches specifically exclude stable RNAs, including rRNA (e.g. Wurtzel *et al*., 2010). We used an SSV1-based reporter gene system in the model archaeon *S. solfataricus* (Jonuscheit *et al*., 2003) to determine whether the *S. solfataricus* core 16S/23S rRNA gene promoter (− 41 to +1) is functional and regulated *in vivo* in response to the growth phase. The core *TF55*α and the

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wild-type *lacS* promoters from *S. solfataricus* were used as controls.

Materials and methods

Plasmid and recombinant viral vector construction

Viral vector pKMSW72 containing the wild-type *lacS* gene in SSV1 was constructed in two steps (primers and plasmids listed in Table 1). First, the *lacS* gene plus 200 bp of upstream DNA was amplified from *S. solfataricus* P2 (DSM1617) DNA via PCR using Pfu DNA polymerase and primers BG840 and BG841, thereby introducing BamHI sites. The BamHI-cut PCR product was ligated into similarly cut pUC28, yielding plasmid pKMSW70. Plasmid pKMSW70 was cut with PstI, dephosphorylated, and ligated to PstI-cut SSV1 to create pKMSW72 (Fig. 1).

Vector pMAD107, containing the core 16S/23S rRNA gene promoter–*lacS* fusion, was constructed in three steps. First, the *lacS* promoter in pKMSW70 was deleting using long-inverse PCR (Clore & Stedman, 2007) using primers pKMSW70MasterF and pKMSW70MasterR. The PCR product was phosphorylated and ligated to produce pMT95. This plasmid was cut with PstI and PacI, dephosphorylated, and ligated to annealed oligonucleotides p16S/23SrRNAF and p16S/23SrRNAR. For annealing, oligonucleotides were incubated at 94 °C for 10 min followed by slow cooling to room temperature. The resulting plasmid, pMAD106, was digested with PstI, dephosphorylated, and ligated into SSV1 cut with PstI to yield pMAD107. In the same manner, primers pTF55αF and pTF55αR were annealed then ligated to pMT95 to produce the *TF55α* promoter-*lacS* construct pMAD109. This plasmid was inserted into PstI-cut SSV1 to create pMAD110. All constructions were confirmed by restriction endonuclease digestion and sequencing of the promoter and part of the *lacS* gene (data not shown). XL-10 Gold supercompetent *Escherichia coli* cells (Stratagene) were utilized for all steps in vector construction.

Transformation of recombinant virus vectors

The pMAD107, pMAD110, and pKMSW72 plasmids, purified from *E. coli* by alkaline lysis (Feliciello & Chinali 1993), were electroporated into *S. solfataricus* PH1 as described previously (Albers & Driessen, 2008). Successful transformation was confirmed by PCR using SSV1-specific primers UnivSSV#7F and UnivSSV#8R (Snyder *et al*., 2004) or B49F and B49R. For UnivSSV#7F and UnivSSV#8R, PCR conditions were as follows: 95 °C 1 min, then 35 cycles, 95 °C, 30 s, 46 °C, 30 s, 72 °C, 1 min, and then 7 min at 72 °C. For B49F and B49R, 95 °C 1 min, then 35 cycles, 95, 60, and 72 °C for 30 s each, then 4 min at 72 °C.

Table 1. Oligonucleotide primers and plasmids used in this study

Primer name	Sequence (restriction endonuclease sites underlined)	Reference
BG840F	5′-GCGGATCCTCTTATTATTAGAATTG-3′	This study
BG840R	5′-GCGGATCCCAAAAGGTACAAA-3′	This study
pKMSW70MasterF	5′-TTTTTTTTTTGACATGTAGTCATTTCCAAATAGCTTTAGG-3′	This study
pKMSW70MasterR	5′-GAGGATCCTCTAGAGTCGACC-3′	This study
p16S/23SrRNAF	5′-GGAAGTTAGATTTATATGGGATTTCAGAACAATATGTATAATGAT-3′	This study
p16S/23SrRNAR	5′-CATTATACATATTGTTCTGAAATCCCATATAAATCTAACTTCCTGCA-3′	This study
pTF55αF	5′-GAGTAAAATTTTTATATAACCTTTTTTTAAGACAGAGTGAT-3′	This study
pTF55αR	5′-TTTAAACTTTTCTATTTCTTTCCTTATAAATCTTTCCCCCTGCA-3′	This study
LacSNtermR	5′-GCGTAAATAATTCCAACTGG-3′	This study
UnivSSV#7F	5′-ATTCAGATTCTGWATWCAGAAC-3′	Snyder *et al*. (2004)
UnivSSV#8R	5′-TCSCCTAACGCACTCATC-3′	Snyder *et al*. (2004)
B49F	5″-ATGGGATGTGCAAAATCTGAGC-3′	This study
B49R	5′-TTAGAACAAATCATTTATTGCTTCTACGAAAGC-3′	This study

Plasmid	Construction	Reference
pKMSW70	*lacS*+pUC28	This study
pKMSW72	*lacS*+pUC28pKMSW70+SSV1	This study
pMT95	pKMSW70 without promoter	This study
pMAD106	pMT95+p16S/23SrRNA/*lacS*	This study
pMAD107	pMAD106+SSV1	This study
pMAD109	pMT95+pTF55α/*lacS*	This study
pMAD110	pMAD109+SSV1	This study

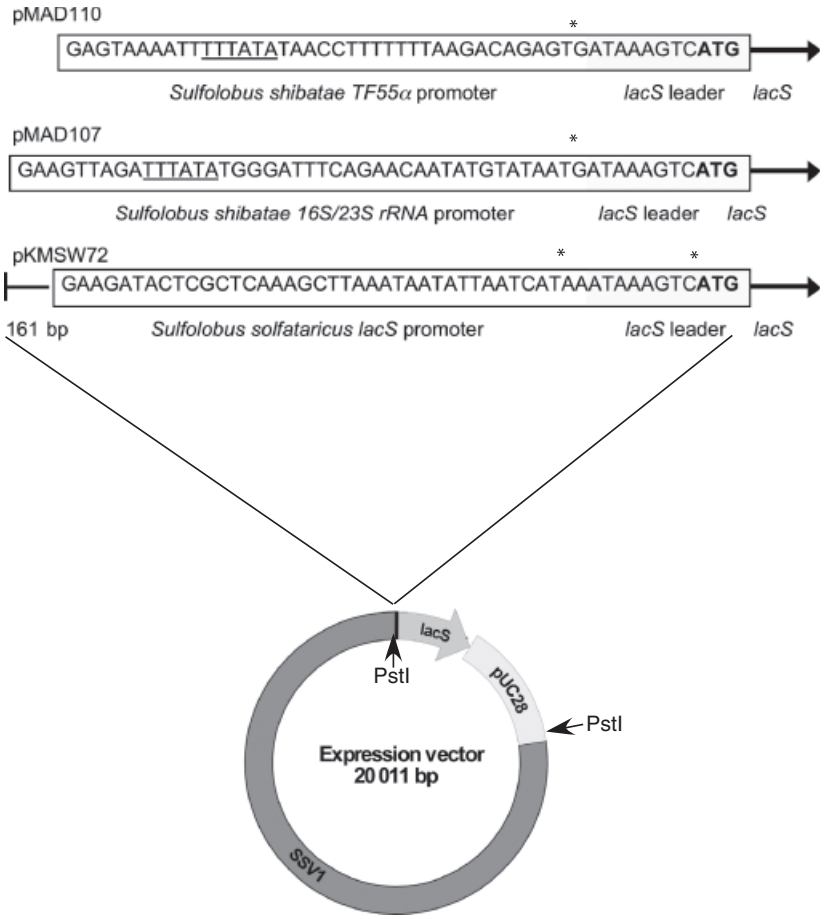

Fig. 1. Schematic diagram of an expression vector with the core promoter sequences. Map of expression vector showing promoter–*lacS* gene fusion, pUC28, and the full SSV1 genome. PstI sites used for subcloning are shown. The known TATA box is underlined for *TF55α* and 16S/23S rRNA gene promoters. Transcription start sites established *in vivo* and *in vitro* are indicated by asterisks (Prisco *et al.*, 1995; Qureshi & Jackson, 1998). The start codon of the *lacS* reporter gene is in bold.

Culture conditions

Sulfolobus solfataricus strains were grown aerobically at 76 °C on plates or in liquid media, both as in Jonuscheit *et al.* (2003). *Sulfolobus solfataricus* strains PH1 and P1 were from Wolfram Zillig's collection. Single *Sulfolobus* colonies containing recombinant viral vectors were isolated by blue-white screening on rich media as described (Schleper *et al.*, 1994). Virus infection was confirmed by PCR. Before all experiments, all strains containing viral vectors were grown to the stationary phase in minimal media containing 0.2% lactose and shifted to room temperature for 2 h to synchronize growth (Hjort & Bernander, 2001). Each culture was then diluted to $OD600_{nm} = 0.05$ in yeast sucrose media, divided into three flasks, and incubated at 76 °C with moderate shaking.

Cell-free extracts

Cell-free extracts were prepared from 8.0 mL of $OD600_{nm} = 0.05$ cultures 1 h after dilution for lag, 2.0 mL of $OD600\,nm = 0.2$ cultures for mid-exponential, and 0.3 mL of $OD600_{nm} = 1.2$ cultures for stationary phase. Cultures were centrifuged for 10 min at 3000 ***g*** and cells were washed once in 1 sample volume of 10 mM Tris pH 8. Cells were resuspended in 400 μL 10 mM Tris pH 8 and lysed by two freeze/thaw cycles of − 80 and 50 °C for 5 min each, and then diluted 1 : 10 in 10 mM Tris pH 8. Protein concentrations of cell-free extracts were determined by micro Bradford assay (Bio-Rad) compared with bovine serum albumin.

β-Galactosidase assay

β-Galactosidase activities were determined by colorimetric endpoint enzyme assay (Jonuscheit *et al.*, 2003). Briefly, 20 μL of each crude cell extract was added to 480 μL preheated 5 mM pNPG in 0.1 M sodium acetate pH 5. After 15 min at 95 °C (optimal temperature for *lacS*; Kaper *et al.*, 2002), 1.0 mL of ice-cold 0.5M $NaHCO_3$ was added and $OD_{405\,nm}$ was measured spectrophotometrically. The amount of enzyme catalyzing the hydrolysis of 1 μmol of pNPG in 15 min at 95 °C is 1 U. The extinction coefficient of pNPG is 15.8 $mM^{-1}\,cm^{-1}$ in sodium acetate pH 5 (Kaper *et al.*, 2002). Extracts from *S. solfataricus* PH1 (*lacS*$^{-}$) and

S. solfataricus P1 (*lacS* wild type) served as negative and positive controls, respectively.

Southern hybridization

Total DNA (Stedman *et al.*, 1999) was extracted from exponentially growing cultures ($OD_{600\,nm} = 0.2–0.3$) of *S. solfataricus* PH1 infected with pMAD107 (*16S/23S rRNAp-lacS*), pMAD110 (*TF55αp-lacS*), or pKMSW72 (*lacSp-lacS*), digested with PstI, separated by gel electrophoresis, transferred and fixed to nitrocellulose membranes. *Sulfolobus solfataricus* PH1 chromosomal DNA and pKMSW72 plasmid DNA were included as size markers. The *lacS* gene was detected by a chemiluminescent probe complementary to the N-terminus of the gene and exposure to X-ray film (Supporting Information, Fig. S2). The vector copy number was determined from multiple exposures by comparing the intensity of the signals from the chromosomal and vector copies of *lacS* using IMAGEQUANT (Molecular Dynamics).

Quantitative PCR (qPCR)

The absolute vector copy number in all cell-free extracts used for growth-phase dependent enzyme assays was determined by qPCR using the QuantiTect SYBR Green PCR kit (Qiagen) on a Strategene iCycler (Table S1). Vector-specific primers B49F and B49R were used at 0.5 μM each. Linearized pKMSW72 quantified spectrophotometically was used as the standard for qPCR quantification (Fig. S3). *Sulfolobus solfataricus* PH1 cell-free extract was the no template control. The qPCR settings were as follows: one cycle at 95 °C for 15 min followed by 40 cycles of 95 °C for 30 s, 60 °C for 1 min, and 72 °C for 30 s. A melting curve was determined after the last cycle to ensure that the measured fluorescence was due to the specific product. The qPCR was performed in triplicate for all samples.

Results and discussion

The 16S/23S rRNA gene and *TF55α* core promoters are sufficient for *S. solfataricus lacS* gene expression *in vivo*

In order to determine whether the core promoters of the 16S/23S rRNA gene (42 bp) and *TF55α* genes (39 bp) were sufficient for expression of the *lacS* reporter gene *in vivo*, we measured β-galactosidase activity in cell-free extracts of *S. solfataricus* PH1 (*lacS*::*ISC1217*) (Schleper *et al.*, 1994) transformed with viral vectors containing the respective promoter–*lacS* gene fusions (Fig. 2). A construct containing 200 bp upstream of *lacS* was used as a positive control. Cell-free extracts from transformants with all three promoters had higher levels of β-galactosidase activity than host background activity, indicating that even the 39/42 bp core promoter sequences were sufficient for *lacS* expression *in vivo* (Fig. 2a). The pattern of β-galactosidase activity did not change significantly when normalized for the relative copy number of the *lacS* reporter gene by Southern hybridization (Fig. 2b).

Previous *Sulfolobus in vivo* gene expression studies using similar SSV1-based reporter gene constructs have shown that 448 bp for the *TF55α* promoter (Jonuscheit *et al.*, 2003) or 241 bp for the *araS* promoter (Lubelska *et al.*, 2006) are sufficient for expression of the *lacS* gene. A 55-bp core promoter plus an 'ara-box' is sufficient for expression of *lacS* when in a pRN2-plasmid-based vector, but not when the 'ara-box' is removed (Peng *et al.*, 2009).

The *Sulfolobus* 42-bp 16S/23S rRNA gene core promoter is sufficient for growth phase-dependent gene regulation *in vivo*

To determine whether the core 16S/23S rRNA gene promoter is regulated *in vivo* in response to the growth phase in *S. solfataricus* PH1, we measured the β-galactosidase activity in *S. solfataricus* PH1 containing the 16S/23S rRNA gene core promoter–*lacS* gene fusion during lag, mid-exponential, and stationary growth phases. Similar constructs with the *TF55α* core and wild-type *lacS* promoters were tested to determine whether regulation is promoter specific. *Sulfolobus solfataricus* strains PH1 and P1 were included as negative and positive controls for β-galactosidase activity, respectively. The β-galactosidase activity did not change drastically between different phases of the growth cycle in wild-type *S. solfataricus* P1 or *S. solfataricus* PH1 containing the *TF55αp–lacS* fusion, indicating that the wild-type *lacS* promoter and the core *TF55α* promoter are not regulated with growth phase (Fig. 3). However, β-galactosidase activity produced by *S. solfataricus* PH1 containing the 16S/23S rRNAp gene–*lacS* fusion increased approximately threefold during exponential growth compared with lag phase (Fig. 3), indicating that the region from −41 to +1 is sufficient for specific regulated transcription in response to entry into exponential growth phase. β-Galactosidase activity due to the core 16S/23S rRNA gene promoter in *Sulfolobus* was 1.7–3-fold lower in the stationary phase than in exponential growth (Fig. 3). The pattern of β-galactosidase activity did not change significantly when normalized for the absolute copy number of the lacS gene by qPCR, indicating that the increase in activity in exponential growth was due to regulation of the 16S/23S rRNA gene promoter, not gene dosage (Fig. 3b). The 42-bp 16S/23S rRNA gene core promoter is the smallest reported regulated promoter for *Sulfolobus*.

These findings are consistent with evidence of upregulation of rRNA transcription during exponential growth in *E. coli* and *Saccharomyces cerevisiae* (yeast) (Nomura, 1999)

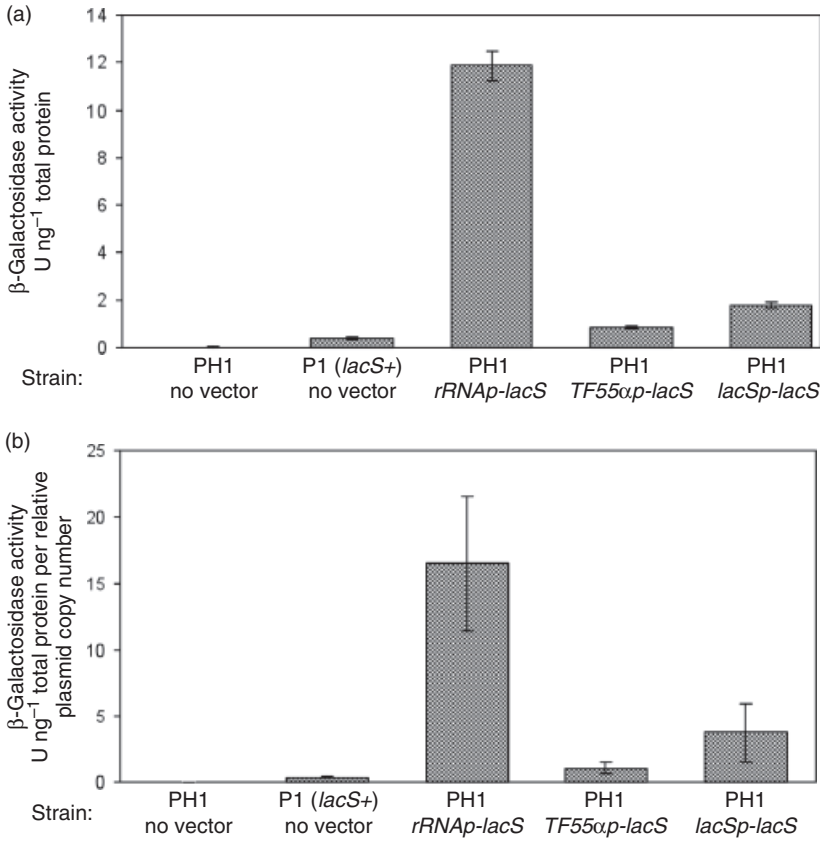

Fig. 2. β-Galactosidase activities in cell-free extracts of *Sulfolobus solfataricus*. (a) Enzymatic activities of cell-free extracts from wild-type *S. solfataricus* P1, *S. solfataricus* PH1 (β-galactosidase *lacS* mutant), and strains of *S. solfataricus* PH1 transformed with promoter–*lacS* gene fusions. (b) Enzymatic activity in crude extracts normalized for relative *lacS* copy number determined by Southern hybridization. Activity and copy number were determined for each biological replicate in triplicate. Mean and SD of three biological replicates are shown.

and with microarray data from halophilic archaea showing that ribosomal protein gene transcription is higher during exponential growth than in the stationary phase (Lange *et al.*, 2007). Moreover, rRNA in crude preparations from *Natronococcus occultus* decreases in the stationary phase (Nercessian & Conde, 2006).

The mechanism for core rRNA promoter regulation in *S. solfataricus* is obscure. The decrease in β-galactosidase activity observed during the stationary phase may be due to growth rate-dependent transcriptional regulation or stringent control in response to decreasing nutrient availability and/or charged tRNAs. The latter has been shown to decrease total stable RNA accumulation in *Sulfolobus* (Cellini *et al.*, 2004).

As in *E. coli* and yeast, it is likely that there are multiple mechanisms contributing to regulation of the *Sulfolobus* 16S/23S rRNA gene operon. There is considerable evidence that archaeal transcriptional regulators interact with core promoters, either binding between or overlapping the TATA box and the transcriptional start site (Peng *et al.*, 2011). *In vivo* and *in vitro* analyses have determined several regulatory regions and the start site of the 16S/23S rRNA gene in *S. shibatae* (Hudepohl *et al.*, 1990; Reiter *et al.*, 1990; Hain *et al.*, 1992; Qureshi *et al.*, 1997). The core promoter sequences necessary for transcription initiation *in vitro* are between −38 and −2 bases relative to the transcription start, identical to those used here *in vivo*. This region encompasses the proximal promoter element (PPE) (an AT-rich sequence −11 to −2 conserved in *Sulfolobus* stable RNA promoters), the TATA box, and several bases upstream thereof (Reiter *et al.*, 1990), later identified as a transcription factor B (TFB) recognition element (BRE) (Qureshi & Jackson, 1998). A weak positive regulatory region between −354 and −190 and a negative regulatory region between −93 and −38 were also found (Reiter *et al.*, 1990). Transcription initiates more efficiently *in vitro* from 16S/23S rRNA gene promoters with purified RNA polymerase, TFB, and the TATA-box binding protein than in cell extracts obtained from stationary phase cells, indicating a negative regulatory factor therein (Qureshi *et al.*, 1997).

Identification of a possible rRNA-specific regulatory motif in the 16S rRNA gene promoter

In order to define motifs in the *S. solfataricus* 16S/23S rRNA gene core promoter possibly important for regulation, the 42-bp sequence was compared with the core promoters from *S. solfataricus* ribosomal protein genes (http://

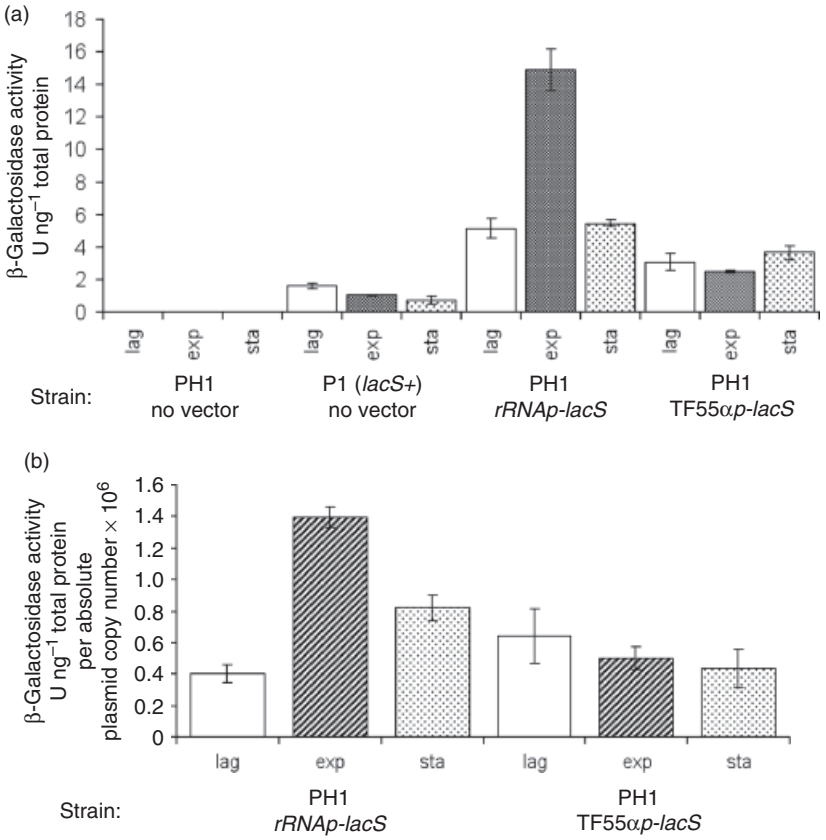

Fig. 3. Growth phase-dependent β-galactosidase activities in cell-free extracts of *Sulfolobus solfataricus*. (a) Enzymatic activities of cell-free extracts from *S. solfataricus* PH1 (*lacS*−), *S. solfataricus* P1, and strains of *S. solfataricus* PH1 transformed with promoter–*lacS* gene fusions during lag (lag), mid-exponential (exp), and stationary (sta) growth phases. (b) Enzymatic activity normalized for absolute *lacS* copy number as determined by qPCR. Each enzymatic assay and qPCR was performed in triplicate. Mean and SD of three biological replicates are shown.

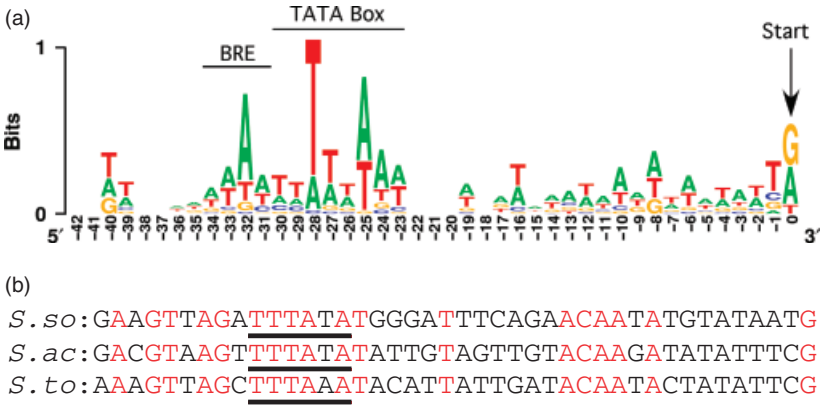

Fig. 4. Sequence comparisons of ribosomal protein core promoters from *Sulfolobus solfataricus* and rRNA core promoters from other *Sulfolobus* species. (a) Relative nucleotide frequencies at each position relative to the transcription start site (arrow) of core promoter of ribosomal protein and RNA genes. Plot was prepared using WEBLOGO (Crooks *et al.*, 2004). Putative BRE and TATA boxes are labeled. (b) rRNA core promoters from *S. solfataricus* (*S.so*), *Sulfolobus acidocaldarius* (*S.ac*), and *Sulfolobus tokodaii* (*S.to*) were aligned to their known (*S.so* and *S.ac*) or predicted (*S.to*) transcriptional starts. Predicted TATA boxes are underlined. Identical nucleotides are highlighted in red.

archaea.ucsc.edu). The only clearly conserved motifs are the TATA box and a potential BRE (Fig. 4a) and these are not conserved with the rRNA promoter (Fig. 4b). Moreover, the BRE sequence is noncanonical (Bartlett, 2005) and the distance between the transcription start site and the TATA box is considerably longer in the rRNA promoters (Fig. 4b), indicating that transcription may be differently regulated between rRNA and ribosomal protein genes. There is also no obviously conserved PPE or downstream BRE, unlike the minimal arabinose-regulated promoters analyzed *in vivo* (Peng *et al.*, 2009) although this region is rich in A/T base pairs and mutations therein reduced activity of the 16S/23SrRNA gene promoter *in vitro* (Hain *et al.*, 1992). To determine whether there was an rRNA-specific regulatory motif, predicted rRNA promoters from other *Sulfolobus* species were compared. The rRNA promoter is identical in *S. solfataricus*, *S. shibatae*, and seven '*S. islandicus*' genomes (Reno *et al.*, 2009), but is less conserved in *S. acidocaldarius*

and *Sulfolobus tokodaii* (Durovic & Dennis, 1994; Kawarabayasi *et al.*, 2001;Fig. 4). Nonetheless, a conserved possible regulatory sequence between −9 and −14, '5′-ACAANA-3′', was identified and remains to be tested.

Changes in β-galactosidase activity are not due to gene dosage changes

To eliminate the possibility that differences in β-galactosidase activity were due to gene dosage effects, the relative or absolute copy numbers of the *lacS* gene in each sample were determined by Southern hybridization or qPCR, respectively. The relative copy number was calculated as the ratio of the signal from the stable vector-borne *lacS* gene to the disrupted chromosomal *lacS* gene (Fig. S2). The average relative vector copy number per chromosome is approximately one (Fig. S2). This is consistent with evidence that the number of plaque-forming units (PFU) per cell of SSV1-based shuttle vectors in *Sulfolobus* cultures remains relatively constant at 1.5 PFU per cell (Stedman *et al.*, 1999). The relative *lacS* copy number was sometimes less than one, suggesting that these cultures contained a mixture of infected and noninfected cells (Fig. S2). When normalized for the relative *lacS* copy number, relative β-galactosidase activities did not change drastically (Fig. 2).

For growth-phase dependent experiments, the absolute copy number of each vector in each culture in all growth phases was determined by qPCR (Fig. S3 and Table S1). Again, this normalization did not drastically change the results (Fig. 3a and b). Hence, gene dosage effects are negligible in cultures grown from single-colony isolates regardless of the different conditions applied (Figs 2 and 3) and are not responsible for changes in the β-galactosidase activity observed at different growth phases.

SSV1-based reporter genes can be used without *pyrEF* selection

Many viruses affect regulation of the host cell's genes in order to redirect the host's machinery to support virus replication. Because little is known about the effects of SSV1 infection on *Sulfolobus*, we cannot rule out that infection with viral vectors caused changes in gene expression. However, growth rates of SSV1-infected cells are very similar to that of uninfected cells (Fig. S1; Frols *et al.*, 2007). Additionally, microarray analyses of stably SSV1-infected compared with uninfected *S. solfataricus* strains indicated minimal transcriptional changes (Frols *et al.*, 2007). It has been reported that similar vectors containing the *lacS* reporter gene were not stably maintained in culture and required the addition of *pyrEF* to stabilize the vector (Jonuscheit *et al.*, 2003; Berkner *et al.*, 2010). We also experienced loss of the vector from primary transformations (not shown). However, isolation of single colonies infected with the recombinant viral vector and subsequent outgrowth in selective media was sufficient for stable vector maintenance (data not shown). Thus, at least under these conditions, the addition of *pyrEF* as a selectable marker is not absolutely necessary and makes the vector somewhat smaller and easier to manipulate. We also did not observe recombination of the viral vector in *S. solfataricus* PH1 cells.

Conclusions

To our knowledge, this is the first experimental evidence for promoter-dependent regulation of the 16S/23S rRNA gene operon in *S. solfataricus* in response to changing cellular conditions and the first evidence for rRNA regulation in hyperthermophilic Archaea in response to growth phase. The severely truncated 16S/23S rRNA gene core promoter is the smallest reported regulated *Sulfolobus* promoter and provides an excellent target for future *in vitro* and *in vivo* studies.

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Supporting Information

Additional Supporting Information may be found in the online version of this article:

Fig. S1. Growth curve of infected and uninfected cells in early and exponential growth.
Fig. S2. Representative Southern blot for copy number determination.
Fig. S3. Typical qPCR standard curve
Table S1. qPCR data.

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